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The Emergence of Modern Neuroanatomy and Developmental Neurobiology

Review

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The Emergence of Modern Neuroanatomy

The decade of the 1970s witnessed a remarkable renaissance in morphological studies of the nervous system, due in large part to the introduction of a variety of new neuroanatomical methods. To appreciate why new methods were needed and to understand how they came to have such an impact, it is necessary to review, albeit briefly, the long history of neuroanatomy and its continuing search for ever more effective ways to visualize the structure of individual neurons and to trace connections between them. And, in retrospect, it is evident that most of the controversies that have beset the discipline, almost from its earliest beginnings in the latter half of the nineteenth century, were mainly due to the limitations inherent in the existing methods or to differing interpretations of what they could or could not reveal. Consider, for example, the long-standing debate as to whether the nervous system is composed of anatomically discrete cells ("the neuron hypothesis") or forms a complex syncytial network ("the reticular theory"). The fact that the great Spanish neuroanatomist Ramon y Cajal should devote his last major work to a defense of the neuron theory (Cajal, 1933), almost a century after the formulation of the cell theory by Scheiden and Schwann, seems today quite astonishing—all the more so when one considers that much of the evidence Cajal was able to marshal derived from his careful studies of material stained by a method introduced more than 50 years earlier by his most outspoken critic, the Italian histologist Camillo-Golgi! It is a measure of Cajal's genius that he recognized that no single method could resolve this issue (or most of the other issues that commanded the attention of neuroanatomists throughout the first 30 years of this century). In the first volume of his masterly *Histologie du Systèm Nerveus de l'Homme et des Vertebres* (1909), he reviewed in some detail the whole range of methods that had been developed from Deiters' manual dissection of single brain cells and Ehrlich's supravital methylene blue staining of nerve fibers, through the many variants of Golgi's silver impregnation technique and a large number of staining procedures including Weigert's method for myelinated fibers, Marchi's technique for staining degenerating myelin, Nissl's use of aniline dyes for cytoarchitectonic studies, and a variety of silver methods for staining axons and their terminals.

Some of these methods (like Ehrlich's supravital staining) were short-lived; but others continued to be used effectively for many decades. For example, much of what we know of the general organization of the human brain is based on Weigert preparations, and neuropathologists continued to use it well into the 1950s to look for the loss of myelin staining in the brains and spinal cords of patients who had suffered from tabes dorsalis or multiple sclerosis. And the Golgi method continued to be used until well into the 1970s, when it was essentially replaced by intracellular labeling methods (see below).

One of the most important discoveries of the early years of neuroanatomy was Waller's (1850) recognition that when an axon is interrupted, the distal segment, including all of its branches, invariably degenerates. This insight was to serve as the basis not only for the Marchi method (since myelin sheaths also break down when axons degenerate) but also for several methods for staining degenerating axons, based on the original Bielschowsky (1902) technique. Of perhaps equal importance was Gudden's (1870) discovery that lesions of the cerebral cortex could lead to profound atrophy of the thalamus, a finding that was soon followed by Nissl's (1913) demonstration that the thalamus was the major subcortical source of afferents to the cortex.

The discovery of anterograde degeneration of axons and their myelin sheaths following damage to the cell bodies or interruption of their projection pathways, and of the retrograde degeneration of cells after damage to their axons or axon terminals, were to remain the cornerstones of experimental neuroanatomy until the early 1970s.

Methods Based on Anterograde Axonal Degeneration

As Cajal had recognized, the study of normal material is useful for analyzing the form of individual neurons, for identifying major cell groups, and for mapping the general disposition of the larger fiber systems of the brain and spinal cord; however, it is severely limited when it comes to establishing connections between widely separated populations of neurons. For this, experimental material was essential. For many years the only available method for tracing degenerating axons was the Marchi technique, despite its obvious and known limitations. Since, as we have seen, the method is based on the staining of the breakdown products of myelin, it was useful only for studying well-myelinated fiber systems, and given the fact that the terminal portions of axons are unmyelinated, it could not demonstrate their precise sites of termination. Furthermore, for reasons that are still unclear, the method was rather capricious and often failed to reveal pathways that were known to exist. Nevertheless, in the hands of several workers, including Polyak (1932), LeGros Clark (1933), and Mettler (1935) it proved to be extremely useful for mapping most of the ascending sensory systems and many of the major cortico-cortical and cortico-thalamic connections.

Having reached its heyday in the 1930s, the Marchi method was largely replaced after World War II by silver impregnation methods that, for the most part, were variants of Bielschowsky's original technique. The first of

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these methods to attract attention was that introduced by Paul Glees in 1946. The Glees method was used quite extensively by the Oxford group to study several different forebrain systems and especially inputs to the monkey hypothalamus. Unfortunately, it was later discovered that in many regions of the hypothalamus and adjoining structures, the brains of normal animals stained by the Glees method often show fragmentary axons that in every respect resemble what, at the time, was described as "characteristic terminal degeneration" (see Cowan and Powell, 1956). This finding, and the appearance of the original Nauta and Gygax (1954) method, soon heralded the demise of the Glees technique.

The introduction of what is familiarly referred to as the Nauta method (and its many subsequent variants) marked the beginning of the renaissance in modern neuroanatomy. What made this method particularly valuable was that it enabled investigators, for the first time, to trace degenerating axons selectively from the site of an experimental lesion to their termination, against a relatively clear background brought about by the active suppression of staining of the surrounding normal neural processes (see Guillery, 1970). The fact that the Nauta methods have been largely replaced by techniques developed for the most part in the 1970s should not obscure the substantial contributions they made to neuroanatomy in the 2 decades between about 1955 and 1975 (Cowan and Cuenod, 1975).

The Retrograde Cell Degeneration Method

The dramatic loss of neurons seen in the principal nuclei of the thalamus after cortical lesions (Nissl, 1913) prompted several workers, most notably LeGros Clark in England and Earl Walker in this country, to systematically examine the thalamo-cortical projection in primates (e.g., LeGros Clark, 1933; reviewed by Walker, 1938). It would be difficult to exaggerate the profound influence these studies had on the entire field of neuroanatomy, even though it became apparent that in many regions of the nervous system the retrograde cell degeneration method was severely limited. In some cases (like most motor neurons in adult animals), interrupting the axons did not lead to the death of the cells, but, at best, to a transient phase of chromatolysis marked by a breakdown of the larger nucleoprotein aggregates commonly referred to as "Nissl bodies" and the movement of the nucleus to the periphery of the cell. In others, like the pyramidal cells of the hippocampus, there is no discernible reaction to axotomy. The reasons for such marked variations in the response of cells to axonal injury is still not known, but in at least one system that was initially thought to be refractory to axotomy (the lateral mamillary nucleus of the hypothalamus) there is evidence that the existence of collateral projections enables the cells to survive the interruption of one or more axonal branches (Fry and Cowan, 1972).

In addition to the variable response of neurons to axotomy, the retrograde cell method presents a number of other complications and interpretative difficulties. For example, in some cases the affected cells do not die but persist in a shrunken state for many months. Cell shrinkage of this kind may be due to axonal injury, but it may also be indicative of an anterograde transneuronal change (of the kind classically seen in the primate lateral geniculate nucleus after eye removal) or of retrograde transneuronal atrophy following the death of the cells upon which the shrunken neurons project. An example of the latter phenonemon is observed after ablation of the cingulate cortex in young rats and rabbits; not only do the cells in the anterior nuclei of the thalamus die, but the cells in the medial mamillary nucleus (that project to the anterior thalamus) also degenerate. And, in very young animals, secondary retrograde transneuronal changes can even be seen in the ventral tegmental nucleus of the midbrain, which projects to the medial mamillary complex (Cowan, 1970). Because of the refractoriness of many types of neurons to axotomy, Brodal (1940) took advantage of the observation that immature cells seem to respond more acutely to injury, to introduce what he termed "a modified Gudden method." This involved placing lesions in very young animals and looking, after a relatively short postlesion survival period, for signs of chromatolysis or cell shrinkage. Brodal used this method for his extensive analysis of the connections of various brainstem nuclei (Brodal, 1957). Unfortunately, attempts to replicate some of his findings using this approach have generally been unsuccessful.

New Approaches to Tracing Connections in the Central Nervous System

Although the retrograde cell degeneration method continued to be used until well into the 1960s, its limitations became increasingly evident and more and more reliance was placed on the use of one or another modification of the Nauta method. But, by the late 1960s, this too was seen to be limited, especially for studies of the developing nervous system and for those regions of the brain where it was virtually impossible to rule out incidental damage to fibers of passage by the causative lesion. Prompted by these considerations, several groups of workers began to explore alternative approaches that were not dependent on degenerative changes and the placing of destructive lesions. It was also hoped that, at the same time, such approaches might permit the chemical characterization of the pathways under study. It is probably not without significance that the emergence of a whole range of new neuroanatomical methods coincided with the emergence of interest in developmental neurobiology. Investigators who were interested in determining when particular central pathways are formed, and how they might be modified later in development, were especially frustrated not only by the vagaries of the axonal degeneration method when applied to the brains of embryonic and early postnatal animals (and especially the rapidity of the degenerative changes in such animals) but also by the necessity of destroying the very structures they wished to study.

The Autoradiographic Method for Tracing Connections

At the time, the most promising approach appeared to be the use of axonal transport to selectively label neural pathways. This approach had been pioneered by Taylor and Weiss (1965), who had shown that one could label the retinal ganglion cells of amphibians by injecting a small amount of a tritium (^{3}H)-labeled amino acid into the eye and could subsequently follow the distribution of the labeled material along the optic nerve fibers to

their termination in the contralateral optic tectum. Droz and Leblond (1963) had earlier shown that following the systemic injection of ^{3}H-leucine in rats, the label first appeared in autoradiographs of the brain in the vicinity of neuronal perikarya (where most protein synthesis occurs), and only later in axons and dendrites, as a result of the active transport of labeled proteins from the cell bodies. That the autoradiographic examination of radioactively labeled and axonally transported proteins could be used as a general method for tracing connections—at least from discrete neuronal populations such as the retina and dorsal root ganglia—was definitively shown by Lasek et al. (1968), who were able to follow the central connections of dorsal root ganglia, in suitably prepared autoradiographs, to their sites of termination in the spinal cord and medulla as readily as in Nauta preparations after dorsal root section. In discussing their observations, they pointed out several potential advantages of this approach. These included: (1) the preservation of the integrity of the system under study and hence the possibility of combining it with functional studies; (2) the fact that the uptake of the labeled amino acid and its incorporation into protein only occurred in neuronal somata obviated one of the more serious problems associated with lesion-based methods, namely the incidental involvement of fibers of passage; and (3) the discreteness of the developed silver grains in the emulsion layer overlying the tissue made it possible to clearly define the course of the labeled axons and their sites of termination. Although they noted that, in principle, the method could be applied to the study of the connections of discrete neuronal populations within the brain and spinal cord, they do not seem to have attempted to do this. And, indeed, one of the first reported attempts at labeling neurons in the brainstem (by injecting a relatively large amount of ^{14}C-leucine and examining various regions of the brain by scintillation spectroscopy) gave no indication of selective axonal transport from the site of the injection (Foulkes and Robinson, 1968).

About this time, two further developments occurred that made the use of autoradiography for studying axonal connections even more attractive. The first was the demonstration by Hendrickson (1969) that the method lent itself readily to electron microscopy and that under the electron microscope it was evident that a significant proportion of the labeled material was distributed to synaptic terminals. The second was the finding that there is more than one component to the axonally transported materials. In particular, the discovery of the relatively rapid transport of labeled proteins (moving with velocities in excess of 100 mm/day; Lasek 1966; Grafstein, 1967), coupled with the finding that much of the rapidly transported material was selectively distributed to axon terminals, suggested that the autoradiographic method could be adapted to identify precisely the sites of termination of neuronal pathways.

Stimulated by these findings, in 1971 my colleagues and I undertook a systematic study of the use of labeled amino acids and autoradiography to trace pathways within the central nervous system. Although our initial experiments were complicated by the excessive background labeling (due to the injection of unnecessarily large amounts of the labeled amino acids), they were sufficiently encouraging to suggest that with refinement the method could be even more effective than the established axonal degeneration methods (Cowan et al., 1972). Since the paper resulting from this study summarized both the advantages and disadvantages of the method, and also provided a detailed protocol for its use, it is hardly necessary to recapitulate its major conclusions here except to note two points. First, it was emphasized that the "digital" quality of the silver grains could be used to quantitate the labeling patterns seen at the sites of termination of certain pathways, and a number of examples of this approach were provided in the report and others were given in later studies (e.g., Gottlieb and Cowan, 1972; Price, 1973). To capitalize on the quantifiable nature of the autoradiographic signal and to relieve the tedium of counting silver grains, computer assisted procedures were subsequently developed; these permitted rapid, automated grain counting and graphic displays of the quantitative data (Wann et al., 1974).

Second, the principal disadvantages of the method were discussed. Leaving aside the somewhat tedious process of preparing autoradiographs, the major disadvantages are that the method cannot be used to study short axonal projections (because of the dense labeling at the site of injection of the labeled amino acid) and, even more importantly, because the label is detected as a collection of silver grains in the emulsion overlying the tissue section, it does not reveal the form of the labeled axons or their terminal ramifications.

One obvious advantage of the method that was not discussed in our original paper, but was soon put to the test, was that it could also be used to analyze the development of neural pathways. Our own studies along these lines were first directed toward determining the time of arrival of retinal axons at the optic tectum of chicks (Crossland et al., 1973) and frogs (Currie and Cowan, 1975) but later were extended to the development of such central systems in the mammalian brain as the hippocampus and dentate gyrus (Fricke and Cowan, 1977). We shall return to this application later when we consider the impact of this and other technical advances on developmental neurobiology.

An interesting and wholly unanticipated finding was the discovery that some of the axonally transported label can be released from the axon terminals and secondarily taken up by the postsynaptic neurons (Specht and Grafstein, 1973). This fortuitous observation was to be particularly useful for analyzing the organization and development of the so-called eye dominance columns in the visual cortex (Hubel and Wiesel, 1977). Although the mechanism underlying the release of the label from the presynaptic axons and its secondary uptake by the postsynaptic cells has not yet been resolved, empirically it is known to occur most readily when large amounts of the labeled precursor are used, when the post-labeling survival period is fairly long, and when the density of the presynaptic terminals of the primary neurons is high (see Hendrickson and Edwards, 1978). Similar transsynaptic labeling of this kind has been found after horseradish peroxidase labeling, but as a tract-tracing method this has not been widely used and has been largely replaced in the past few years by the postsynaptic propagation of viruses (reviewed by Sams et al., 1995).

Methods Based on Retrograde Axonal Transport

It was several years after the discovery of the anterograde transport of materials from the neuronal soma along axons and dendrites before the significance of transport in the reverse direction was fully appreciated. That intraaxonal materials were moving in a retrograde direction was implicit in the early experiments in which peripheral nerves were cut or ligated, and an accumulation of material was observed on both sides of the transection or ligature (e.g., Dahlström, 1965). And, although its significance was not fully grasped at the time, it is noteworthy that as early as 1925 it had been proposed that certain neurotropic viruses could track back along nerve fibers from the periphery to the brain (Goodpasture, 1925).

In the late 1950s and mid-1960s, several different lines of experimental evidence made it clear that axonal transport was, indeed, bidirectional. Among the critical observations were the in vitro demonstration that organelles such as mitochondria and various types of vesicle could move from the growing tips of axons back toward the soma (e.g., Hughes, 1953), and the accumulation of noradrenaline (Dahlström, 1965) distal to a nerve crush, which suggested that this retrograde movement was a significant physiological phenomenon. Credit for recognizing that retrograde transport could be useful as a neurobiological tool should perhaps go to Krister Kristensson (1970), who used Evans blue–labeled albumen to demonstrate that exogenous proteins could be transported from an injection into a leg muscle of a mouse back to the bodies of the innervating motor neurons in the spinal cord. Later, he and a colleague (Kristensson and Olson, 1971) showed that the enzyme horseradish peroxidase (HRP) could similarly be transported retrogradely from muscles to the related motor neurons.

That the retrograde transport of HRP could be used as a general method for identifying the cells of origin of central neural pathways was first proposed by the LaVails in 1972 (LaVail and LaVail, 1972), and some of the critical parameters relating to this use were more fully documented in several later papers by Jennifer LaVail and a succession of colleagues (reviewed by LaVail, 1978). The essential simplicity of this method—involving as it does the injection of a small volume of a concentrated solution of HRP into a region of the brain and, after a period of a few hours to 1 or more days, fixing the brain, cutting frozen sections, and reacting the enzyme with a suitable substrate (usually diaminobenzidine)—had considerable appeal (especially to nonneuroanatomists) and made the "HRP method" one of the more widely used neuroanatomical tracing methods throughout the 1970s and early 1980s. It is no exaggeration to say that it completely replaced retrograde neuronal degeneration as the method of choice for determining the cells of origin of neuronal pathways.

It is not necessary here to consider the many variants of the basic method that were introduced, including the use of other molecules coupled to HRP (like wheat germ agglutinin), various other markers that were tried, and various procedures introduced to restrict the spread of the marker at the injection site—all of which have had their proponents at different times. Suffice it to say that the most significant development was the recognition that HRP (and other similar labels) were not exclusively transported retrogradely, but could also be used to demonstrate—at times quite exquisitely—sites of axonal terminations in the brain or spinal cord (reviewed by Lynch et al., 1974; Spencer et al., 1978).

The Use of the Plant Lectin Phaseolus vulgaris *Leucoagglutinin (PHA-L) to Trace Connections*

Despite its widespread use as a label to trace connections, both retrogradely and anterogradely, HRP—alone or conjugated with the plant lectin wheat germ agglutinin—was often less than optimal. In particular, it was usually necessary to inject a fairly large amount of the enzyme in order to get adequate uptake and transport, and this made it difficult to define precisely the effective site of the injection. And although occasionally the retrogradely labeled neurons were sufficiently filled with the reaction product (giving a Golgi-like appearance), more commonly the labeled cells had only a diffuse, granular appearance. Similarly, the method seldom displayed the fine morphology of anterogradely labeled axons and their terminals. Prompted by these considerations, in the early 1980s my colleagues Chip Gerfen and Paul Sawchenko considered a number of alternative labeling procedures and finally settled on the plant lectin *Phaseolus vulgaris*–leucoagglutinin (or phytohemagglutinin-L) as the most suitable (Gerfen and Sawchenko, 1984).

The PHA-L method, as it is known, has several advantages as an anterograde labeling procedure. When the lectin is introduced iontophoretically, injection sites as small as 50–100 μm can be produced, and the neurons at the injection site are usually completely filled so that even structures as small as dendritic spines can be identified. Axons of passage do not seem to take up the lectin, but axons that are anterogradely labeled can be easily followed to their termination where fine terminal branches and presynaptic boutons are well defined. Furthermore, since the transported lectin is visualized either by immunofluorescence, using commercially available antibodies, or by immunoperoxidase staining, the method can be used in conjunction with other techniques such as the retrogradely transported fluorescent dyes described below. Because of the clarity with which the PHA-L method reveals central pathways and their terminations, in the past 15 years or so it has largely replaced most of the earlier methods.

Intracellular Labeling Methods

Like labeling with HRP, the PHA-L method is only really useful for studying long axonal projections; neither approach can be used to study relatively short axons or locally distributed axon collaterals. For this the Golgi technique, in one of its many variant forms, had long been the method of choice. Despite its notorious capriciousness (especially in inexpert hands), the fact that the technique usually impregnates only a small proportion of the neurons in any given region but those that are stained can often be visualized in their entirety—including, in the best preparations, the axons and axon collaterals—made it invaluable. Indeed, virtually all we knew of the organization of dendritic arbors and of "local circuit neurons" until the late 1960s and 1970s had come from the analysis of Golgi-impregnated material. This

situation changed dramatically some years after the introduction of glass micropipettes for intracellular recording when it was shown that one could iontophoretically fill neurons with either a fluorescent dye (like Procion yellow; Stretton and Kravitz, 1968) or with opaque materials such as cobaltous sulphide (Pitman et al., 1972) or the HRP reaction product (Muller and McMahon, 1976; Snow et al., 1976). It soon became apparent from the use of these markers that both dendritic arbors and axonal branching patterns are often considerably more complex than can be seen in all but the very best Golgi impregnations. And the fact that intracellular filling could be done in tissue slices, and combined with electrophysiological recordings *from the same cells*, has helped to make this approach the method of choice for analyzing the detailed morphology of individual neurons, including, if necessary, their synaptic organization as seen in electron micrographs of appropriately prepared sections.

Until the early 1970s, observations on Golgi-impregnated material and on individually labeled neurons were essentially qualitative. The complex three-dimensional structure of most neurons made it difficult to quantitatively assess their morphology. To overcome this, a variety of indirect methods had been proposed, based generally on such stereological approaches as counting the numbers of dendritic segments that intersect a given reference landmark or the numbers found within circles of given radius centered on the cell body (Sholl, 1953; Valverde, 1970). To provide more precise quantitative data, my colleagues at Washington University and I developed a semi-automated computer system that accurately recorded the full three-dimensional structures of neurons and permitted their display from any required spatial angle (Wann et al., 1973; Cowan et al., 1975). Since that time, a number of similar computer systems have been introduced, including some that are fully automated.

The Use of Fluorescent Dyes to Study Long Collateral Projections

Golgi-impregnated and intracellularly labeled neurons are invaluable for studying local axonal branching patterns, but are of little help if one is interested in more distal collateral projections. For this, a method first introduced by Kuypers and his colleagues, based on the retrograde labeling of neurons with various fluorescent dyes, has proved to be extremely useful. In principle, the method is quite simple: if a neuron projects to two or more widely separated regions, it should be possible to double (or triple) label them by injecting suitable fluorescent dyes into each region to which the cells send collateral projections. Optimally, the dyes should have widely different emission spectra or be selectively distributed to different cellular components such as the nucleus or cytoplasm. That this worked in practice was first demonstrated by van der Kooy et al. (1977), who, by injecting different fluorescent dyes into the thalamus of the two sides and double labeling cells in the mamillary nuclei, showed that single neurons in certain of the mamillary nuclei project bilaterally to the anterior nuclear complex of the thalamus.

My colleagues and I were especially interested in using this method to determine whether the widespread projections of the various fields of the hippocampal formation were due to the collateral branching of individual neurons or to a heterogeneity among the neurons that comprise each field. We were able to resolve this issue by making localized injections of one dye (such as "True Blue") into one projection site and similar injections of another dye (e.g., bisbenzimide or "Nuclear Yellow") into a second projection field. If care was taken to ensure that the injections were in the appropriate topographical regions of each projection field, virtually all of the neurons at the site of origin of the two projections could be shown to be double labeled (see Swanson et al., 1980, 1981). Since our previous attempts to resolve this issue using other methods had proved to be rather equivocal, the ineluctible evidence provided by the retrogradely transported fluorescent dyes was especially gratifying.

As the dyes that we and others used seemed to be nontoxic and could often remain within the labeled neurons without being degraded for days if not weeks, it occurred to my colleagues Dennis O'Leary and Brent Stanfield that they could also be used to study the developing nervous system and especially to determine if changes occurred in the patterns of connections during development (see below).

Fluorescence Histochemistry and Immunocytochemical Methods

The development in the 1960s of methods to identify biogenic amines in tissue sections by Falck, Hillarp, and their colleagues (see Falck, 1962; Falck et al., 1962) marked the beginning of a new era in neuroanatomy. Hereafter, the field would no longer be limited to mapping connections between different neuronal populations: it was now possible to identify populations according to the neurotransmitter(s) they utilize and, in a word, to begin to think of a "chemical neuroanatomy" (see Fuxe et al., 1970; Moore and Loy, 1978). In the late 1970s, this approach began to be broadened by the growing availability of antibodies suitable for immunocytochemistry, to include not just the biogenic amines but a wide range of conventional neurotransmitters, neuropeptides, various enzymes, and other proteins with distinctive distributions among neuronal groups. In time, this approach was to be complemented by in situ hybridization techniques that permit the identification of virtually any transcribed gene within neurons and by techniques for selectively labeling the receptors (or various subunits of the receptors) of almost every class of neurotransmitter, neuropeptide, or neurotrophin. However, with the exception of the Falck-Hillarp method, these later developments were only perfected in the 1980s and 1990s and so lie outside the scope of this review.

The Emergence of Modern Developmental Neurobiology

It would be misleading to imply that the advances in neuroanatomical methods discussed above were responsible for the renewed interest in neural development that occurred in the 1970s. But, at the same time, it is clear that they certainly paved the way for the rapid progress in our understanding of the development of the vertebrate central nervous system that marked the decades of the 1970s and 1980s and that continues

unabated to the present time. To appreciate the relationship between the two advances, one has only to consider what at the time was commonly called "neuroembryology." Neuroembryology had had a long and distinguished history going back at least to the 1890s and, at a descriptive level, much of what we now take for granted was clearly set out by the great zoologists and anatomists of that era, including most notably Wilhelm His and Ramon y Cajal. It is to His that we owe our general view of the sequential changes in the form of the developing brain from the closure of the neural tube to its definitive adult appearance. And it is to Cajal and his brilliant use of the Golgi method that we owe our understanding of the development of individual neurons, the appearance of growth cones at the ends of their processes, and, most astonishingly, the microscopic appearance of virtually all regions in the developing brains of each of the major vertebrate classes. Based on his careful observations, Cajal also put forward a number of remarkably prescient hypotheses concerning axon guidance, synapse formation, and the elimination of misplaced neurons and aberrant connections, as well as the latent capacity for regeneration and plasticity in the mature nervous system (Ramon y Cajal, 1909, 1911).

It is somewhat surprising, given Cajal's lead, that for more than 3 decades much of the work on the developing central nervous system, including the seminal experimental studies of such distinguished scholars as Ross Harrison, Samuel Detwiler, and Paul Weiss (and, initially, even Roger Sperry) relied either on behavioral responses or were analyzed anatomically at a rather gross morphological level. For example, during this period, one of the major issues was the relationship of the central nervous system to the "periphery" as studied by limb or eye removal, of limb, ear, and eye rotations, or the addition of supernumerary limbs, and xenografts of large eyes into smaller-eyed species. In nearly every case, the experimental material was analyzed only in terms of the overall size of the affected regions rather than on an examination of their intrinsic structures. On reading the literature of this period, one is struck by the contrast between the conceptual brilliance of the experiments and their interpretation and the disappointing quality of the anatomical work-up of the material.

This situation changed rather rapidly after World War II. When Rita Levi-Montalcini joined Victor Hamburger's laboratory, she brought with her a number of neuroanatomical techniques, including De Castro's modification of Cajal's original silver impregnation technique (Ramon y Cajal and De Castro, 1933). Levi-Montalcini's work on the development of the chick auditory and vestibular systems (1949) and her joint work with Hamburger on the effects of early limb ablation on the development of dorsal root ganglia (Hamburger and Levi-Montalcini, 1949) set the stage for the dramatic discoveries of nerve growth factor (NGF) and for much of the later work on what came to be known as "naturally occurring" or "programmed cell death" (see Levi-Montalcini, 1966).

In retrospect, perhaps the first and most important contribution of the 1970s to vertebrate developmental neurobiology was the clear articulation of the issues that needed to be addressed. None of these issues was wholly new, but their formulation was to set the agenda for the field for the ensuing 2 decades. The critical issues, more or less in the order in which they arise during the development of the vertebrate nervous system, were as follows. (1) What determines how the ectoderm along the dorsal midline is set aside to form the neural plate and the adjoining neural crest? (2) How is cell proliferation in the central and peripheral nervous systems regulated? (3) How do neurons migrate from their sites of generation to their definitive locations in the mature nervous system? (4) How do neurons and glial cells acquire their distinctive morphological features, their unique physiological properties, and their ability to transmit and receive signals to and from other cells? (5) How do developing axons "find their way" to their appropriate target regions and, within those regions, selectively form synapses with the correct cell types? (6) What "errors" occur during cell migration and axonal pathfinding, and how are these corrected? (7) Why do so many neurons die during development and what brings about their death? And finally, (8) what role does activity play in shaping neural circuitry?

It is obviously impossible, given the present space constraints, to do more than briefly touch upon most of these topics. Two, however, merit special attention: the mechanism of axonal pathfinding and target recognition and the role of cell death as a regulatory mechanism in the development of essentially all neuronal populations in the vertebrate nervous system.

Neural Induction

Little progress was made during the 1970s in our understanding of the initial process of *neural induction*. Indeed, it is only in the past 7 or 8 years that some of the key molecular players in this process have been identified and a new paradigm put forward to account for the differing fates of somatic and neural ectoderm (reviewed by Weinstein and Hemmati-Brivanlou, 1997; Harland, 1997). Similarly, until methods had been developed for identifying and cloning neural genes, no significant progress could be made in understanding neuronal and glial cell differentiation or the molecular mechanisms involved in axon guidance, target identification, or selective cell death. At a descriptive level, however, considerable progress was made, and this prepared the groundwork for the rich harvest of discoveries that followed the introduction of molecular genetic approaches to the problems.

Cell Proliferation in the Vertebrate Nervous System

Among the first advances in this period was the use of ^{3}H-thymidine labeling to map the patterns of cell proliferation in the brain and spinal cord (reviewed by Sidman, 1970). The large body of work, using this approach, can be summarized by saying that (1) cell proliferation in each neuronal population occurs over a well-defined period of some days or weeks, (2) that proliferation in nearly every neural structure that has been examined is patterned both spatially and temporally (in the sense that different regions and different populations of cells within the structure are generated in an orderly temporal sequence), and (3) the times at which cells withdraw from the proliferation cycle (sometimes referred to as their "dates of birth") are closely correlated with their

final locations and their functional characteristics. A single example of this last finding will suffice to make these general points (although, of course, the detailed patterns vary from region to region). In the mammalian neocortex, cell proliferation in posterior regions tends to begin earlier than in more anterior areas; deeper cortical neurons are generated earlier than more superficially located cells (that is to say there is a distinct inside-out pattern of neurogenesis [Rakic, 1974]); and neuronal phenotype (including both the physiological properties and the types of connections formed by the cells) can be correlated with the time the cells become postmitotic (even when the cellular architecture of the cortex is disordered, as in the mutant *reeler* mouse [Stanfield et al., 1979; Lemon and Pearlman, 1981]).

Neuronal Migration

Since most neurons in the vertebrate central nervous system are generated within the neuroepithelium lining the ventricular wall or in the adjacent subventricular zone, they must all undergo at least one phase of migration before coming to reside in their definitive locations. In the 1970s, it became clear that most young neurons migrate radially outward from the ventricular or subventricular layers, along the surface of radially disposed glial cell processes (see Rakic, 1975). Evidence that this was more than a fortuitous relationship was descriptive and largely indirect, but was supported by the claim that the radially oriented processes of Bergmann glial cells in the cerebellar cortex degenerated at an early stage in the mutant mouse *weaver*, in which many granule cells failed to migrate from the external to the internal granular layer (Rakic and Sidman, 1973). At the same time, it was also known that many classes of neurons and neuronal precursor cells (like the granule cells of the olfactory bulb) could migrate over long distances in a direction orthogonal to the prevailing radial glial cell processes. It was more than a decade before this issue could be analyzed more directly and some of the molecular players in the process identified (Hatten and Mason, 1990). During this period evidence was also adduced, from experiments using retroviruses as lineage markers, that the progeny of single stem cells in the mammalian brain may be widely dispersed and not confined to a narrow radial column as earlier described (reviewed by Cepko et al., 1997).

Neuronal Differentiation

Although the postmitotic cells that migrate away from the ventricular or subventricular zones can be shown by cytochemical markers to be nerve cells, most neurons seem only to acquire their full complement of neuronal features after reaching their definitive locations. This is certainly true of their morphology, as several studies of the appearance of different classes of neurons clearly demonstrated. And as Cajal had amply documented more than half a century earlier, the dendritic arbors and the patterns of axonal branching of neurons usually undergo a considerable degree of remodeling as the cells mature. Particularly striking in Golgi-impregnated cells is the early appearance of many long, spine-like processes, most of which disappear as mature synapses begin to form on the cells' dendritic shafts and spines (where these occur). Considerable interest centered also on the degree to which the characteristic dendritic trees of neurons like cerebellar Purkinje cells, cortical pyramidal cells, etc., are shaped by their local environments or are due to purely intrinsic factors (reviewed by Cowan, 1979). Attempts to answer this question by growing dissociated neurons in low density cell cultures (Banker and Cowan, 1979) or by examining the dendritic branching patterns of neurons in specialized regions, such as the stellate cells in the cerebellar cortex or the neurons that form the barrels in layer IV of the somatosensory cortex of rodents (Woolsey et. al., 1975), suggest that both intrinsic and extrinsic factors are involved.

In those neurons that could be readily impaled with microelectrodes (including various cells in both invertebrates and vertebrates), it could be shown that the electrophysiological properties of the cells do not emerge full-blown but gradually evolve over a period of some hours or days (Spitzer, 1979; Llinas and Sugimori, 1979). The actual sequence of events seems to vary among different types of neurons, but one fairly common one is characterized by the appearance first of only passive electrical properties and electrical coupling among the cells, followed by the emergence of Ca^{2+} action potentials of long duration; this is followed by an intermediate phase, during which the cells display characteristic Na^{+} spikes superimposed upon the slower Ca^{2+} action potentials, and finally, the replacement of Ca^{2+}-mediated responses by Na^{+} action potentials. More detailed analysis had to await the application of patch-clamping and whole cell recordings, and more recently the availability of molecular probes for the relevant ion channels, neurotransmitter synthesizing enzymes, and receptors. But one of the most interesting findings during the 1970s concerned the ability of peripheral autonomic neurons to switch from one neurotransmitter to another. This was clearly shown in vitro when sympathetic ganglion cells were maintained for several days in culture (Patterson, 1978) but was also evident when neural crest tissue was transplanted from the thoraco-abdominal segments to the head region (Le Douarin, 1976).

Axonal Pathfinding and Target Recognition

The central issue in developmental neurobiology, of course, concerns how the complex patterns of connections within the central and peripheral nervous systems are formed. Not surprisingly, this issue had attracted the attention of almost every student of neural development since before the turn of the century. And, once again, it was Cajal who first clearly articulated the key questions: What causes the axon of a neuron to emerge from one or the other pole of the cell? How does the axon grow toward its target area through a myriad of other cells and process? How do axons that cross to the opposite side of the brain or spinal cord recognize and respond to cues at the midline and on the contralateral side? How do axons identify their appropriate target cells, and how do they come to form synapses only upon the appropriate parts of those cells?

I shall not attempt to review in any depth the varieties of experimental approaches that were used during the 1970s to examine these issues, except to say that conceptually they were largely influenced by the earlier work of Ross Harrison and his colleagues and especially by the imaginative studies of Roger Sperry, which culminated in his clear formulation of what he termed the

"chemoaffinity hypothesis" (Sperry, 1963). Since Sperry's studies have informed almost all later work on this topic, it is instructive to reexamine how, as a result of his ingenious experiments and critical thinking, the entire field was transformed. As he pointed out, "for a long time it was believed that the developing embryo [spun] out a random, diffuse, unstructured, essentially equipotential transmission network . . . leaving behavior, function practice, experience, learning and conditioning to mold and shape the fiber pathways into a functionally adaptive communication system" (Sperry, 1965). Since this view was most vigorously and dogmatically propounded by his one time mentor, Paul Weiss, it took Sperry some years to discern how deeply flawed it was and how it flew in the face of an ever-increasing body of evidence to the contrary (reviewed by Hunt and Cowan, 1990).

Sperry began by carefully defining the central ideas underlying the "functionalist" position (as it came to be known) and several hypotheses that flowed from it. If the hypothesis were correct, Sperry argued, it should be possible to show: (1) that developing or regenerating nerves are interchangeable, (2) that central and peripheral innervation is nonselective, and (3) that anatomically maladaptive connections can be corrected by function and experience. It is impossible here to do justice to the wide range of experiments that Sperry carried out to test these hypotheses using a variety of neural systems and many different species, from goldfish and newts to frogs and rats. But some indication of the broad strategies he adopted and the types of experiment he and his colleagues conducted is needed to demonstrate the pivotal role he played between the early 1940s and the mid-1960s.

Sperry's first strategy was to surgically impose upon an animal a set of anatomically (and behaviorally) maladaptive connections and then to test its ability, over time, to either correct the inappropriate connections or to replace them with functionally adaptive circuitry. His second strategy was to provide growing axons with a choice of targets—some appropriate, some inappropriate—and then to assess whether they were selective in their choice of the appropriate target rather than the inappropriate. His initial experiments were designed to critically test Weiss' view that muscle innervation is nonselective and that adaptive motor activity is secondarily brought about by a mechanism that Weiss termed "resonance"—a rather ill-defined form of matching of motor neuron activity to the distinctive activity of the muscles they innervate. Sperry's experiments involved, first, transposing the flexor and extensor muscles to the ankle joint (Sperry, 1940) and, second, cross-uniting the peroneal and tibial nerves that innervate antagonistic muscles (Sperry, 1941). If the functionalist hypothesis was correct, normal foot movements should, in time, be restored. In fact, even after several months, the animal's movements remained maladaptive and "showed no adjustments, either immediate by means of reflex regulation or gradual by means of learning or conditioning" (Sperry, 1940).

Later, Sperry took advantage of the regenerative capacity of the optic nerve in fish and amphibians to examine the specificity (or otherwise) of central connections. After establishing that normal vision was established some weeks after simple transsection or crushing of the optic nerve, in a now classic set of experiments he transected the nerve and rotated the eye by 180°. After sufficient time had elapsed for regeneration to occur, he then tested the orienting and striking responses of the animals to a suitable lure placed in different parts of its visual field, and examined the movements of their eyes when they were subjected to a standard optokinetic test using a rotating drum (Sperry, 1943). For as long as the animals were maintained, their orienting and striking behavior was always misdirected by 180° and their optokinetic responses were similarly maladaptive. This proved to be true also when he subsequently transposed the left and right eyes. In these experiments (depending on how the transposition was done) the animals responses were misdirected either dorsoventrally or anteroposteriorly (Sperry, 1945). From all of these findings, Sperry concluded that regenerating axons always grow back selectively to their normal target areas within the contralateral optic tectum. Moreover, as he was to show in another set of experiments designed to test the restoration of color vision in teleost fish, the regenerating axons are able to identify not only the correct region of the tectum, but also to selectively synapse upon the appropriate class of tectal neurons (Sperry, 1948).

In the late 1940s, he and his student Nancy Miner extended this work to the cutaneous sensory system to again test whether or not the periphery exerted a regulatory influence on its innervation. The best known of the various experiments they conducted involved transposing portions of the (white) belly skin to an area on the (dark) backs of frogs and vice versa. When the transplanted belly skin was touched, the animals made wiping movements directed toward the belly and, conversely, its wiping movements were directed toward the back when the transplanted back skin (now on the belly) was stimulated (Miner and Sperry, 1950). Again Sperry was to conclude that "[these] results . . . eliminate mechanical guidance and functional adaptation as the factors responsible for the neural organization mediating cutaneous local sign" and "the patterning of synapses between sensory and central neurons is [best] explained in terms of our chemoaffinity theory of synapse formation" (Sperry and Miner, 1949).

Among the last group of developmental studies Sperry carried out, before turning to the effects of surgical interruption of the corpus callosum, were several that had a lasting influence on all later work on retino-tectal connections. They are also of interest here because they provided the first direct, anatomical evidence that regenerating retinal axons can selectively regrow to their appropriate target regions within the tectum even after such dramatic manipulations as surgically misdirecting the fibers into the "wrong" branch of the optic tract (Arora and Sperry, 1962). Particularly striking were the experiments in which various parts of the retina were ablated, leaving (in different cases) only the central region or the anterior or posterior half of the retina intact. Some weeks after crushing the optic nerve, sections of these brains were stained with Bodian's protargol/silver method. These showed very clearly that in every case the regenerating axons had bypassed inappropriate regions of the tectum to reach their normal projection

fields (Attardi and Sperry, 1963). Moreover, since the retinal fibers re-sorted themselves a short distance beyond the crushed region of the nerve, and always entered the appropriate branch of the optic tract, Sperry concluded that "not only the details of synaptic association within terminal centers, but also . . . the route by which the fibers reach their synaptic zones . . . [are] . . . subject to regulation during growth by differential chemical affinities" (Attardi and Sperry, 1963).

In its definitive form, Sperry's chemoaffinity hypothesis implied that the cells in each neuronal population acquire, at an early stage in their development, a distinct set of chemical markers that defines their position within the population as a whole and at the same time determines the spatial pattern of the connections they establish. To the objection that this might require an almost infinite number of different molecular markers, Sperry suggested, with remarkable prescience, that a spatially graded set of relatively few molecules could in principle suffice to specify the topographic location of any neuron within a two- or three-dimensional array (Sperry, 1963).

Sperry's ingenious exploitation of the projection of the retina upon the optic tectum made this the favored system for study by several groups in the 1970s. For example, Gaze and his colleagues in the U. K., and Jacobson and his student Hunt in the U. S., repeated many of Sperry's experiments (but using electrophysiological rather than the behavioral and anatomical methods Sperry had used to map the retino-tectal projection). The objectives of these studies were to determine (among other things) when, during development, the retinal projection becomes "specified," how it progresses during development, and how it is organized following either partial excision of the tectum or when the fibers from two half-retinae are directed into a single tectal lobe (see Jacobson, 1978, for an extensive review). In our own work, many of the newer neuroanatomical methods were used in both frogs and chicks, to examine some of the same issues (see Currie and Cowan, 1975; Crossland et al., 1975). Without elaboration, it will suffice to say that the general conclusions from all of these studies were consistent with the chemoaffinity hypothesis and, at the same time, served to rule out a variety of alternative proposals, e.g., that the spatial array of the retinal ganglion cells is strictly maintained by their axons as they traverse the optic nerves and tracts, or that their time of arrival at the tectum determines the topographic pattern of its innervation. It should be noted that although the final pattern of the retino-tectal projection found in these experiments was extremely precise, it could be shown that the initial innervation of the tectum is a good deal less exact (see below). It was also evident that, at least in amphibians and fish, the system is capable of considerable regulation, as this term is understood by developmental biologists. Thus, a complete but very compressed map of the retina could be reformed even when a significant portion of the tectum was ablated (reviewed by Cowan and Hunt, 1985).

The most serious obstacle to the general acceptance of the chemoaffinity hypothesis was the complete absence of evidence for molecules that display a graded distribution of the type Sperry had envisaged. This hiatus in our knowledge began to be filled toward the end of the 1970s and early 1980s. Only two examples of how this has been approached will be cited here. The first was the identification by Trisler et al. (1981) of a monoclonal antibody that recognized an antigenic determinant with a smoothly graded distribution across the dorsoventral extent of the chick retina. The second is the elegant series of studies by Bonhoeffer and his colleagues on the ability of retinal axons to grow in vitro onto alternating stripes made up of cell membranes derived from the "appropriate" and "inappropriate" target region of the chick optic tectum, culminating last year with the identification of members of the Eph family of receptors as likely candidates for such molecular markers (Bonhoeffer and Huf, 1980, 1982; and Drescher et al., 1997).

Cell Death during Neural Development

A good deal of attention was paid in the 1970s to the effects on the development of various neuronal populations of surgically removing their normal targets. The study of "center–periphery relations," as it is sometimes referred to, had a long history going back to the classical studies of Ross Harrison and his students in the 1920s and 1930s; but the beginning of the modern period can be traced to Victor Hamburger's first study of the effects of wing bud ablation on the developing motor columns of the chick spinal cord (Hamburger, 1934). What distinguished this study from its many predecessors was the careful microscopic examination of the spinal cord and the finding that, several days after removal of the limb bud, the related motor column had to all intents and purposes disappeared. Influenced as he was by the work of his mentor Hans Spemann, and by contemporary thinking about the role of inductive processes in development, Hamburger was inclined to interpret his findings as evidence for a failure of differentiation of motor neurons (from a large pool of undifferentiated cells in the ventral horn) in the absence of an appropriate inductive signal from the developing wing. In support of this notion, he later reported that early limb ablation had no effect on mitotic activity in the relevant segments of the spinal cord (Hamburger, 1948). Some years later, Rita Levi-Montalcini came across Hamburger's 1934 paper and determined—despite the appalling conditions in war-torn Italy—to repeat his experiments. By examining the spinal cords of chicks with limb bud ablations, Levi-Montalcini found that motor neurons do in fact develop in the absence of the limb and migrate normally into the presumptive motor column. However, shortly thereafter the motor cells degenerate and, as Hamburger had found, leave no trace of their former existence (see Levi-Montalcini, 1981). As a similar large-scale degeneration was also observed in the corresponding sensory ganglia, Levi-Montalcini concluded that the effect of limb bud ablations was not upon the differentiation of either the related sensory or motor neurons but rather on their survival. Shortly after the end of the war, she was invited by Hamburger to join him in St. Louis, and together they carried out their now classical study of the development of chickk sensory ganglia (Hamburger and Levi-Montalcini, 1949) that was to serve as the paradigm for similar studies for almost 2 decades. In addition to confirming Levi-Montalcini's conclusion that cell proliferation, migration, and differentiation of sensory neurons was completely independent of the limb, and that the cell loss that

occurs after limb ablation is a secondary phenomenon, their paper was important for recognizing that a proportion of the neurons in the sensory ganglia of normal chicks also degenerates. Although there had been earlier reports of occasional degenerating cells in the developing nervous system (see Cowan, 1973), this was the first study to suggest that normally occurring cell death might be a general feature of neural development.

Surprisingly, comparatively little attention seems to have been paid to the finding of what was later termed "programmed cell death," until it was observed in a number of neural systems for which precise quantitative data could be obtained. In retrospect, this is now recognized as one of the more important advances made during the 1970s (reviewed by Oppenheim, 1981).

Without detailing the many studies of center–periphery relations that occurred in the 1970s, it will suffice to summarize the general conclusions that emerged both from experiments involving the complete or partial ablation of specific target fields and from studies in which it was possible to effectively expand the target region. In a word, in every system that has been studied from this point of view, it has been found that manipulating the periphery has no discernible effect on the earliest stages of development. The precursor cells proliferate normally, and the young neurons migrate to their appropriate locations and show all the initial features of neuronal differentiation. But in every case there is a period of naturally occurring cell death, which generally involves between 30% and 70% of the cells that initially assemble to form the relevant ganglion, nuclear group, or cortical layer. Since the timing of these cell deaths coincides with the more massive (or total) cell death that occurs after ablation of the population's target projection field, it is likely that the two phenomena are causally related. And this, in turn, suggests that neuronal survival is critically dependent on the availability of trophic materials that are normally produced in only limited amounts by cells within the neuron's projection field. In support of this conclusion was the later finding by Holliday and Hamburger (1976) that enlarging the projection field—in the case of motor neurons in the lumbosacral region of the spinal cord, by introducing a supernumerary limb—could rescue at least a proportion of the cells that would otherwise die.

Elmer Bueker, a student of Hamburger's, had earlier attempted to influence the growth of the motor cell column by transplanting into chick embryos tissue from two sarcoma cell lines (Bueker, 1948). The transplanted tissue was found to have no effect on the motor columns, but as Levi-Montalcini observed when she repeated this experiment in 1952, the tumors did have a profound effect on the neighboring sympathetic ganglia and on the outgrowth of postganglionic axons (Levi-Montalcini, 1952). This observation marked the beginning of the extraordinary saga that led finally to the isolation of nerve growth factor (NGF), which for many years was the only known neuronal trophic factor (Levi-Montalcini, 1966). The fact that NGF acted on certain sensory ganglion cells, as well as sympathetic neurons, allowed Brunso-Bechtold and Hamburger (1979) to directly confirm that NGF is taken up by sensory fibers and retrogradely transported to their cell bodies, as the trophic hypothesis required. They also found that making exogenous NGF available to the fibers (by injecting it into the developing limb) could substantially reduce the amount of cell death in the corresponding ganglia. It remained for later work in the 1980s and 1990s to identify a number of additional neurotrophins and their receptors and to show that they play a similar role in other parts of the nervous system and are important also for neuronal differentiation and the stabilization of connections (reviewed by Reichardt and Fariñas, 1997). It was also some time before the molecular mechanisms involved in the actual death of cells in the vertebrate nervous system would be established, and for this the insightful work of Horvitz and his colleagues on programmed cell deaths in the nematode would prove to be seminal (reviewed by Agapite and Steller, 1997). It would have been difficult to predict in the 1970s how work on naturally occurring and induced cell death in the developing nervous system would lead to the recognition of programmed cell death or "apoptosis" (as it is now called) as a phenomenon of general importance in nearly all biological systems. Its significance for the nervous system, however, was fairly clear by the late 1970s: it appears to serve two broad purposes. First, it provides for the quantitative matching of the size of each neuronal population to that of its target; second, it is responsible for the elimination of neurons that project aberrantly to the "wrong" target field or to an inappropriate region within that field (reviewed by Cowan et al., 1984). It should be mentioned also, for the sake of completeness, that it was later shown that, at least in some systems, the survival of neurons is also dependent on their receipt of an adequate afferent input.

Developmental Errors and Their Elimination

The availability of methods for retrogradely labeling neurons early in development made it possible for the first time to recognize that during the phase of cell migration, some neurons become misplaced and end up outside the general boundaries of the nuclear group or cortical layer to which they belong (Clarke and Cowan, 1975). Later, it was shown that early in development many neurons project aberrantly to inappropriate targets or to topographically inappropriate regions within their target field (Cowan et al., 1984). As noted above, both of these classes of "developmental errors" are largely corrected by the death of the ectopic or aberrantly projecting neurons, during the period when connections are being refined.

The Refinement of Connections during Development

It had been generally assumed, mainly from studies on the innervation of limbs, that the patterns of connections formed during development are, from the beginning, rather precise. However, during the 1970s it became clear that this is often not the case and that there is normally a period during which the initial pattern of connections is progressively refined. Perhaps the most striking evidence for this phenomenon was the demonstration that in monkeys there is, at first, considerable overlap in the projection of the inputs from the two eyes (by way of the lateral geniculate nucleus) to layer IV of the visual cortex. Later, the inputs from the two eyes become progressively separated until finally the characteristic "eye dominance columns" are established

(Rakic, 1977). Several other examples of this type of connectional refinement were reported over the next decade, leading to the general conclusion that "pruning" and "sharpening" are general (if not universal) features in the development of connections in the vertebrate nervous system. The fact that the refining process could be prevented by functionally blocking activity in the projecting neurons (for example, by prolonged treatment with tetrodotoxin throughout the refinement period) suggested that although activity plays no discernible role in the initial patterning of connections, their later refinement is an activity-dependent process (Fawcett et al., 1984). Exactly how activity shapes neuronal connectivity was not evident at the time, although selective visual deprivation experiments indicated that in some way patterned stimulation and competition between the inputs from the two eyes was involved (LeVay et al., 1980). It would not be until the 1990s that this issue began to be understood in molecular terms (Shatz, 1997).

In 1970, Redfern had shown that initially muscle fibers are innervated by a number of different axons and that over a period of several days all but one of the innervating axons is eliminated. That polyneuronal innervation of this kind was a fairly widespread phenomenon was later demonstrated for autonomic ganglion cells by Lichtman (1977; see also Lichtman and Purves, 1980) and for the climbing fibers of the cerebellum by Crepel et al. (1976). It is still not clear why the terminals of one axon persist in these situations while the others degenerate or are withdrawn, but Lichtman's work made it clear that it is not because the postsynaptic cell is unable to sustain a large number of synapses: at the same time that multiple innervation is being eliminated in the submandibular ganglion, each surviving axon can significantly increase the number of synapses it forms on the related postganglionic cell.

Except in rather special cases like the cerebellar climbing fibers, it was difficult to demonstrate the selective elimination of axon terminals in the central nervous system. However, it was clear (as Cajal had initially proposed) that in many systems appreciably more connections are formed than persist in the mature nervous system. One of the clearest demonstrations of this was provided in 1977 by Innocenti and his colleagues (Innocenti et al., 1977). In mature animals, callosal connections between the two cerebral hemispheres are sharply limited to certain cortical fields and not others (e.g., the visual cortex); however, at an earlier stage in their development, callosal connections are found throughout the entire hemisphere (including the visual cortex). At the time, it was not clear whether the refinement of the callosal projection was brought about by the selective death of the neurons in certain cortical areas, or by the elimination of callosal axon collaterals given off by neurons that project elsewhere within the ipsilateral hemisphere. This issue could only be resolved by the use of the double-labeling procedures described above. These clearly established that in those areas of the cortex that in adults lack callosal connections, there are, at earlier stages, neurons that send collaterals to the contralateral cortex. Most (and possibly all) of these cells survive beyond the period when the callosal projection is refined, but the collaterals they send into the corpus callosum are selectively eliminated (O'Leary et al., 1981). Later work by O'Leary and his colleagues was to show that the selective elimination of axon collaterals occurs in other cortical projection systems, and has led to the notion that from the point of view of their subcortical connections, all cortical areas are initially equivalent. Only later do the regional differences become apparent as different axon collateral projections are selectively eliminated (O'Leary and Stanfield, 1989).

It is hoped that the foregoing account of some of the discoveries made during the 1970s will serve to illustrate how the advances made during that decade built on the work of the previous 70 or more years and, at the same time, provided the basis for much of the research of the ensuing 18 years. Certainly, from the point of view of developmental studies, the decade of the 1970s was a transitional period between the era of purely descriptive studies to the modern era, in which molecular and cellular approaches are beginning to elucidate the underlying mechanisms that shape and regulate the development of the nervous system. In retrospect, one might argue that this transition would not have occurred as rapidly or as effectively were it not for the new neuroanatomical methods that were developed during the same period.

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